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Diphenyl diselenide and analogs are substrates of cerebral rat thioredoxin reductase: A pathway for their neuroprotective effects

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ABSTRACT

Thioredoxin reductase (TrxR) isoforms play important roles in cell physiology, protecting cells against oxidative processes. In addition to its endogenous substrates (Trx isoforms), hepatic TrxR can reduce organic selenium compounds such as ebselen and diphenyl diselenide to their selenol intermediates, which can be involved in their hepatoprotective properties. Taking this into account, the aim of the present study was to evaluate the hypothesis that ebselen, diphenyl diselenide and its analogs (4,4′-bistrifluoromethyldiphenyl diselenide, 4,4′-bismethoxydiphenyl diselenide, 4.4′-biscarboxy-diphenyl diselenide, 4,4′-bischlorodiphenyl diselenide, 2,4,6,2′,4′,6′-hexamethyldiphenyl diselenide) could be substrates of rat brain TrxR. In the presence of partially purified rat brain TrxR, diphenyl diselenide, bismethoxydiphenyl diselenide and bischlorodiphenyl diselenide (at 10, 15 and 20 μM) stimulated NADPH oxidation, indicating that they are substrates of brain TrxR. In contrast, ebselen and bistrifluoromethyldiphenyl diselenide, that have been previously demonstrated to be substrate of hepatic TrxR, were not reduced by rat brain TrxR. The results presented here suggest that diphenyl diselenide can exert neuroprotective effects by mimicking glutathione peroxidase activity and also via its reduction by TrxR. However, ebselen was not reduced by brain TrxR, indicating that the neuroprotective properties of this compound is possibly mediate by its glutathione peroxidase-like activity.

Oxidation is a fundamental part of life and aerobic metabolism. However, excessive free radicals production, as well as low antioxidant defenses can be harmful to living cell. In effect, reactive oxygen and nitrogen species can oxidize macromolecules (protein, DNA, lipids and carbohydrates), which can lead to cell dysfunction. Therefore, oxidative stress can be involved in the development of chronic degenerative diseases, including neurological diseases [4,10,12,15,24]. Of particular pharmacological significance, experimental studies have indicated that antioxidants may reduce oxidative damage associated with different pathological conditions [2,3,7,20].

Selenium (Se) is an integral component of about 25 selenoproteins, including critical antioxidant seleno-enzymes; namely, the isoforms of glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) [1,13,19,21,23]. Selenium is normally found as a selenocysteine residue and its selenol group is a softer and stronger nucleophile than its sulfur (cysteine) analog. Consequently, the selenol group is expected to attack toxic electrophile species more efficiently than thiol analogs [8,9,19]. Maintenance of full GPx and TrxR activity by adequate dietary selenium supply has been proposed to be useful for the prevention of several cardiovascular and neurological disorders [23].

Of particular therapeutic significance, organoselenium compounds can mimicry endogenous antioxidant enzymes, such as GPx or can be metabolized by TrxR to form selenol intermediates [6,16,25,26] that can imitate the function of the antioxidant selenoenzymes [14,18,19]. For instance, ebselen and diphenyl diselenide have antioxidant, anti-inflammatory and neuroprotective properties [16–20,22] that can be mediated, at least in part, by their *in vivo* metabolism to selenol intermediates [18,19].

Recently we have demonstrated that diphenyl diselenide and some analogs are substrates of rat liver mammalian TrxR [6], which is in accordance with previous studies from the laboratory of Holmgren [25,26], which has demonstrated that ebselen and its diselenide were good substrates of *Escherichia coli*, human placenta and calf thymus TrxR. Of potential pharmacological significance, the reduction of these substrates by mammalian TrxR isoforms can generate selenol intermediates that could imitate the role of some antioxidant selenoenzymes, such as GPx and TrxR [6]. In effect, Holmgren and collaborators have indicated that the TrxR-like (i.e., the ability of TrxR to form the selenol intermediate of ebselen) is more important than the glutathione peroxidase-like activity of ebselen [25,26]. In analogy, the beneficial effects of diphenyl diselenide and its analogs can also be mediated via their reduction catalyzed by the liver TrxR [6].

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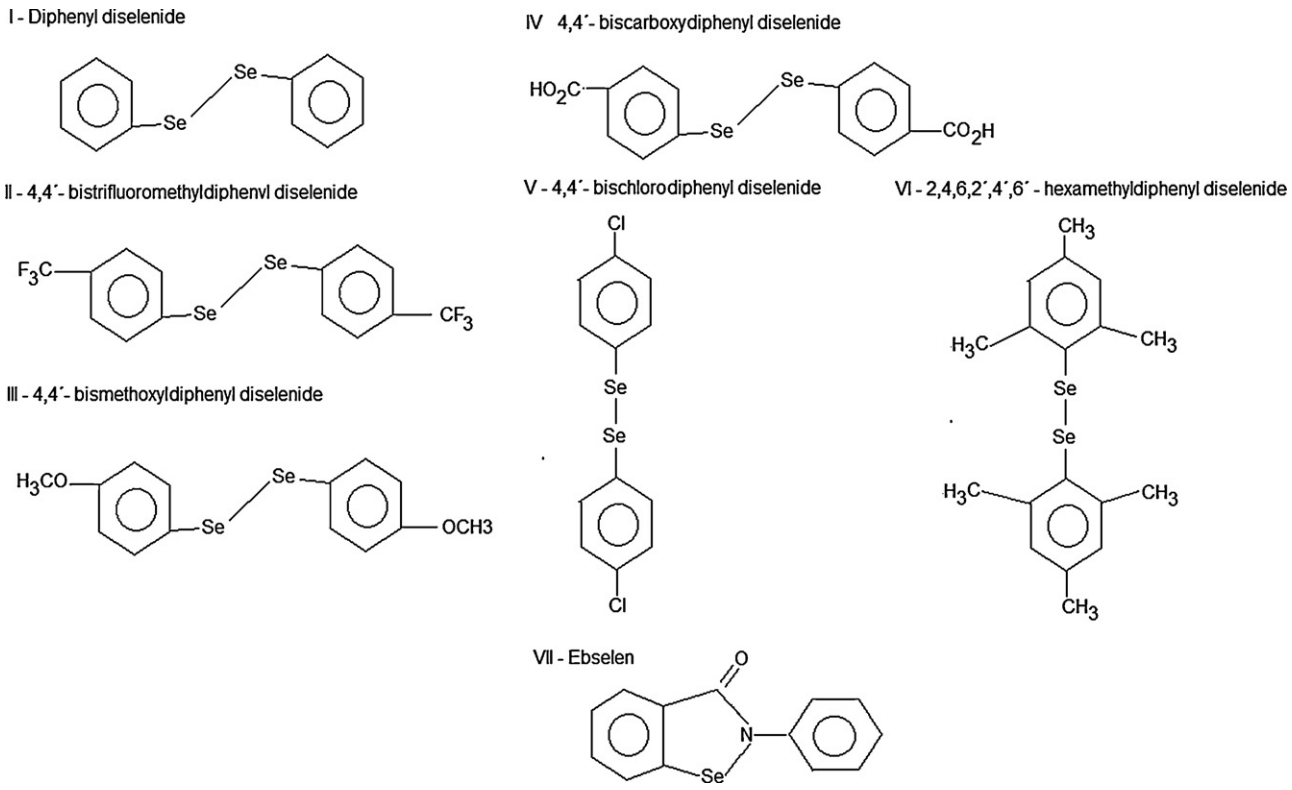


Fig. 1. Structures of diselenide compounds and ebselen.

However, to the best of our knowledge, the potential reduction of diphenyl diselenide and ebselen by cerebral TrxR enzymes has not been previously investigated. In effect, the reduction of ebselen (or its diselenide) and of diphenyl diselenide by brain TrxR can be an important molecular mechanism involved in their neuroprotective properties. We therefore aimed to evaluate the hypothesis that diphenyl diselenide and its analogs (4,4′-bistrifluoromethyl-diphenyl diselenide, 4,4′-bismethoxy-diphenyl diselenide, 4,4′-biscarboxy-diphenyl diselenide, 4,4′-bischloro-diphenyl diselenide, 2,4,6,2′,4′,6′-hexamethyl-diphenyl diselenide) (Fig. 1) could be substrates for rat brain TrxR. Furthermore, we have done a comparative study with ebselen, a prototype organoselenium compound that has been used in clinical trials with borderline success [5,18,19,22].

All Chemicals were of analytical grade and were obtained from Sigma Aldrich, Merk or Fluka. TrxR from rat brain was purified essentially as described by Hill et al., 1997 [11].

TrxR activity was determined according Zhao and Holmgren, 2002 [25]. Activity was performed in a buffer containing 50 mM Tris–HCl, 1 mM EDTA, pH 7.5, 100 μL of TrxR (8–10 μg protein/mL) and 100 μM of NADPH. The quantity of enzyme was chosen based on previous experiments, in which trials were conducted with concentrations of 2.5, 5.0, 7.5, 10, 12.5, 15.0 and 20 μg of the partially purified protein/mL of reaction medium and we have observed a linear reaction from 5.0 to 12.5 μg/ml when diphenyl diselenide and 4,4′-bischloro-diphenyl diselenide were used as substrate (data not shown). Enzyme reaction was started with the addition of organoselenium compounds. Since ebselen and bistrifluoromethyl diselenide did not stimulate NADPH oxidation in the presence of brain TrxR, we also tested concentrations of 2.5, 5, 7.5, 30 and 50 μM (data not shown).

Statistical analysis were performed using three-way (type of compound) × (concentration) × time ANOVA. Univariate analyses followed by Duncan's multiple range test were performed where appropriate.

In the presence of partially purified brain mammalian Trx Reductase (TrxR), diphenyl diselenide, bismethoxydiphenyl diselenide and bischlorodiphenyl diselenide (at 10, 15 and 20 μM) stimulated NADPH oxidation, indicating that they are substrates of brain rat TrxR (Fig. 2A, C and E). However, ebselen and bistrifluoromethyl diselenide did not stimulate NADPH oxidation in the presence of partially purified rat brain TrxR. Consequently, ebselen and bistrifluoromethyl diselenide are not substrate of cerebral TrxR obtained from rats (Fig. 2B and G).

Here we have investigated the ability of diphenyl diselenide and its analogs (4,4′-bistrifluoromethyl-diphenyl diselenide, 4,4′-bismethoxy-diphenyl diselenide, 4,4′-biscarboxy-diphenyl diselenide, 4,4′-bischloro-diphenyl diselenide, 2,4,6,2′,4′,6′-hexamethyl-diphenyl diselenide) and ebselen to serve as substrate of rat cerebral and hepatic TrxR enzymes. Of note, in our recent study [6], we observed that diphenyl diselenide, 4,4′-bistrifluoromethyl-diphenyl diselenide, 4,4′-bismethoxy-diphenyl diselenide, 4,4′-bischloro-diphenyl diselenide and ebselen were substrate of hepatic TrxR; in contrast, here we have observed that ebselen and bistrifluoromethyl-diphenyl diselenide were not reduced by cerebral TrxR. The differential expression of TrxR isoforms and the existence of alternative splice variants of TrxR in different mammalian tissues or cells [1] could explain, at least in part, why ebselen and bistrifluoromethyl-diphenyl diselenide were not substrate of rat brain TrxR.

In accordance with our previous results [6], here we have confirmed that ebselen is substrate of rat hepatic TrxR and stimulated NADPH oxidation (Table 1). Furthermore, as previously observed [6], diphenyl diselenide was six times more active than ebselen as a substrate of hepatic TrxR (Table 1). The results presented here suggest that diphenyl diselenide could exhibit neuroprotective properties by mimicking the activity of glutathione peroxidase and also via its reduction catalyzed by rat brain TrxR enzymes (Scheme 1A). However, ebselen was not a good substrate of rat brain TrxR, indicating that its neuroprotective properties can be

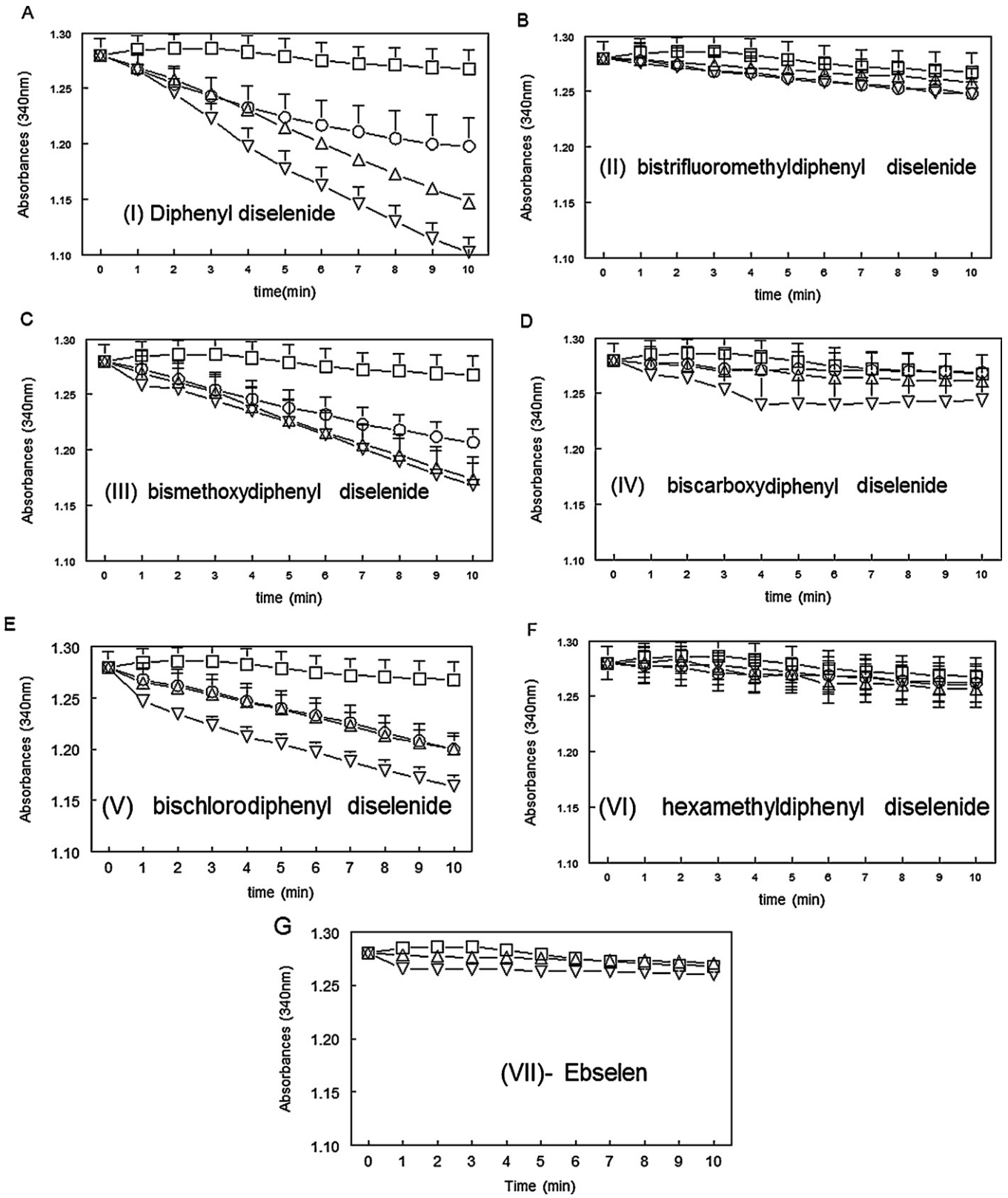
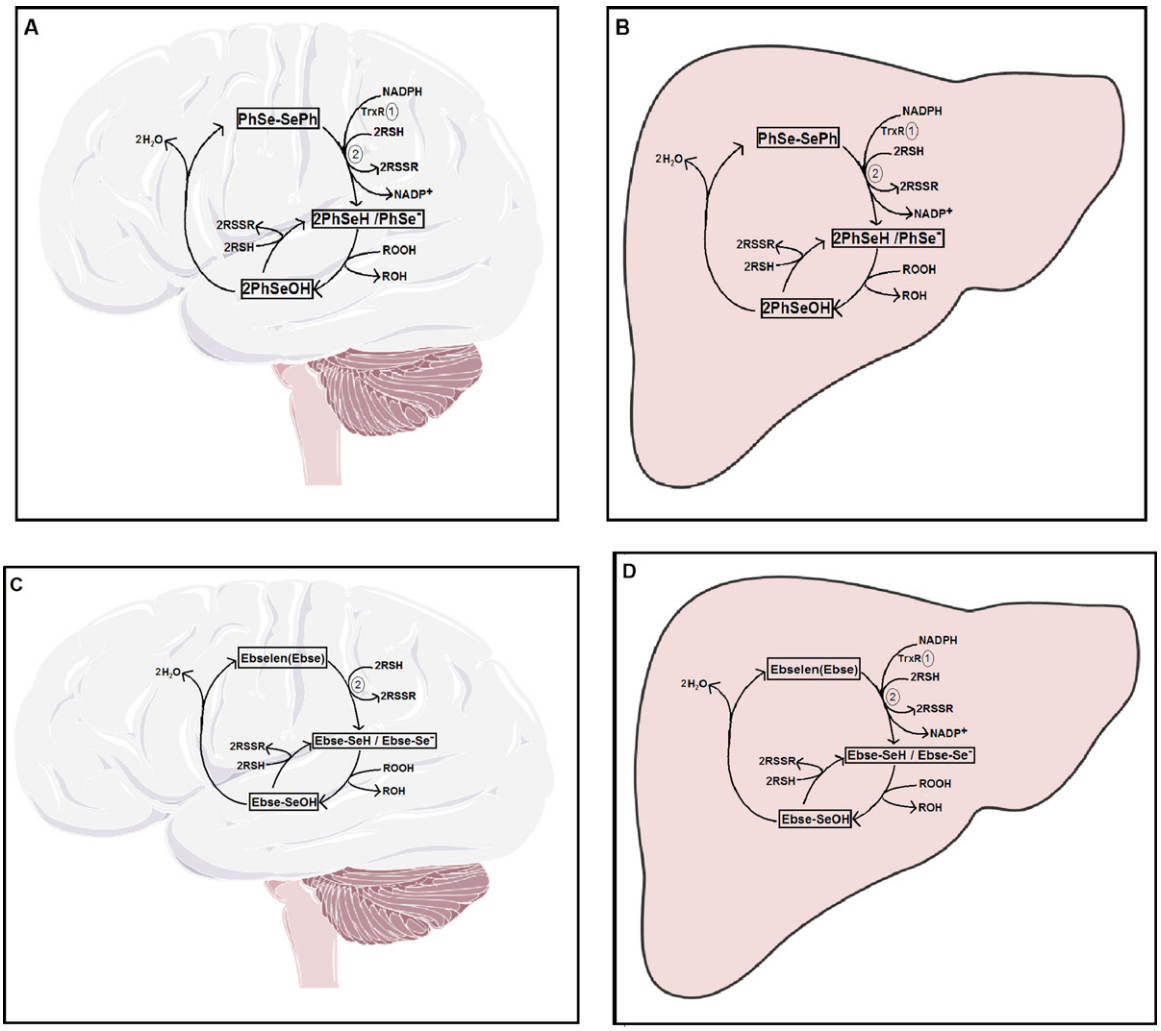


Fig. 2. Reduction of diphenyl diselenide compounds and ebselen I (A), II (B), III (C), IV (D), V (E), VI (F), and VII (G) by cerebral mammalian thioredoxin reductase (TrxR). Enzyme was mixed with a medium containing 50 mM Tris–HCl, 1 mM EDTA, pH 7.5 and then the reaction was started by adding NADPH (final concentration 100 μM). 0 μM (□), 10 μM (○), 15 μM (△), and 20 μM (▽) of diselenide compounds or ebselen. Statistical analyses were performed by three-way ANOVA (7 compounds × 4 concentrations × 12 time points). Data analysis yielded a significant type of compound × concentration × time interaction $F(180, 1680) = 11.7$; $p < 0.000001$, which indicates that the consumption of NADPH was dependent on the concentration, on the type of compound and on the sampling time.



Scheme 1. Mechanisms for neuroprotective (A and C) and hepatoprotective (B and D) effects of diphenyl diselenide (PhSeSePh) and Ebselen (Ebse). Two pathways are indicated: (1) indicates the TrxR catalyzed formation of selenophenol (the selenol intermediate of diphenyl diselenide, PhSeH (A and B) and the selenol of ebselen, Ebse-SeH (D). (2) Indicates the thiol-peroxidase-like pathway, where endogenous reduced thiols can directly reduce diphenyl diselenide and ebselen to their respective selenol intermediates (A–D).

associated predominantly with its thiol peroxidase-like activity (Scheme 1C). In contrast, the hepatoprotective effect of both compounds can occur via the two pathways (Scheme 1B and 1D). However, the extent of operation of GPx-like or the TrxR-like (i.e., the NADPH reduction of ebselen or diphenyl diselenide catalyzed by TrxR) pathway *in vivo* is unknown. Consequently, a better understanding of the antioxidant properties and, the potential pharmacological and therapeutic use of these agents could be greatly improved with the determination of the extent that each pathway works in different tissues.

Table 1
Comparative Reduction of Diphenyl diselenide and Ebselen by Hepatic and Cerebral TrxR.

ΔAbs 340 nm (NADPH oxidation/min) × 10^3	Control	Diphenyl diselenide	Ebselen
Liver	1.00 ± 0.57	86.00 ± 4.91	14.31 ± 0.70
Brain	1.05 ± 0.14	11.60 ± 0.80	0.60 ± 0.05

Data are expressed as mean ± SEM for 3 independent experiments. Two-way ANOVA (2 (tissues: liver or brain) × 3 (compounds: control, diselenide or ebselen) yielded a significant main effect of tissues ($F(1,12) = 307.4$; $p < 0.001$), main effect of compounds ($F(2,12) = 323.6$; $p < 0.001$), and a significant interaction tissues × compounds ($F(2,12) = 188.5$; $p < 0.001$).

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